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GRANT NUMBER DAMD17-94-J-4039

TITLE: Predoctoral Training in Breast Cancer Biology and Therapy

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REPORT DATE: August 1998

TYPE OF REPORT: Final

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 1998	Final (1 Sep 94 - 31 Aug 98)

4. TITLE AND SUBTITLE
Predoctoral Training in Breast Cancer Biology and Therapy

5. FUNDING NUMBERS
DAMD17-94-J-4039

6. AUTHOR(S)
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, MD 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

Substantial improvements in the prevention, detection and treatment of breast cancer require the addition of new laboratory-trained investigators. The objective of this predoctoral training program was to attract new investigators into the field of breast cancer research and to provide them with an interdisciplinary predoctoral training experience focused on this malignant disorder.

The Program comprised a graduate faculty of 34, who are members of 12 departments at the University of Pittsburgh and are interested in breast cancer and graduate education. An infrastructure has been developed to allow graduate students entering any of the 7 Institutional Ph.D. granting programs to be selected for membership in this Predoctoral Training Program. The Predoctoral Training Program in Breast Cancer Biology and Therapy requiref a core of 32 credits of formal course work, including participation in an ethics course, a weekly seminar/research-in-progress series and a newly constructed interdepartmental course on Breast Cancer Biology and Therapy. A minimum of 72 credits with a cumulative grade point average of at least 3.0 has to be obtained prior to graduation. Student retention and progress was monitored by the Breast Cancer Training Grant Executive Committee.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Predoctoral Training, Molecular Biology, Pharmacology, Biopsychology, Immunology, Epidemiology, Genetics, Pathology, Biochemistry, Breast Cancer	32
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

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____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

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✓ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

8/31/98

PI - Signature Date

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E. PROPOSAL BODY

1. Program Vision

Grants to support training of predoctoral students are usually given to a particular training program in an established scientific discipline or a subdiscipline, rather than for training in a specific disease entity or in a particular model system. Thus, training grants are relatively common in pharmacology, virology, immunology, epidemiology, psychology or biochemistry, regardless of the specific problems various investigators from these disciplines are addressing. What distinguishes these discipline-based predoctoral training programs from our **Training Program in Breast Cancer Biology and Therapy** is our multidisciplinary approach and the focus on a specific and important disease. The overall philosophy of this Training Program was to identify qualified graduate students in existing basic life science departments, to educate them in the problems associated with breast cancer and to enhance their research capabilities in this field. Our Training Program expanded the existing pool of investigators studying breast cancer. Moreover, the program encouraged currently funded investigators to focus on breast cancer as an area of study.

Our Graduate **Training Program in Breast Cancer Biology and Therapy** was initiated in 1994, facilitated by this award. In the last four years, during the tenure of the award, we have supported 12 graduate students in seven different departments (Pathology, Epidemiology, Environmental Health, Molecular Genetics and Biochemistry, Pharmacology, Neurobiology, Biology) from three different schools ((School of Medicine, Graduate School of Public Health (GSPH) and Faculty of Arts and Science (FAS)) at the University of Pittsburgh. Six students were supported in the first two years of the grant and another six in the last two years. These students were in the third and fourth, or fourth and fifth year of their Ph.D. thesis research. At that point, they had mastered the intricacies of their various disciplines and they, as well as their mentors, had directed their research project towards solving a problem relevant to breast cancer. We provided support for two years, during which time research projects were initiated and completed. In order to provide the best possible training environment for these students, we organized a seminar series, journal club, yearly retreats and various symposia, which further strengthened our program and extended it to actively participating postdoctoral fellows and faculty. We now have a well organized and well recognized training program.

The **Training Program in Breast Cancer Biology and Therapy** encourages successful and enthusiastic investigators in various disciplines to address questions related to breast cancer, in part by giving them students who can be educated while participating in the project. The program emphasizes a multidisciplinary approach to the problem, educates a significant number of young investigators on the importance of breast cancer research and raises intrinsically interesting biological questions regarding breast cancer.

2. Program Goals

a) To recruit qualified predoctoral students in the **Training Program in Breast Cancer Biology and Therapy.**

b) To educate students in the fundamental principles of breast cancer pathobiology and therapy.

c) To monitor and evaluate the progress of the enrolled students and mentor them in their future career choices.

d) To organize program activities, such as seminar series and journal clubs, for increased interaction of the student trainees with postdoctoral fellows and faculty interested in breast cancer.

3. Training Environment

3.1 The Institution: The University of Pittsburgh, founded in 1787, is one of the oldest institutions of higher education in the United States. At present, it comprises 16 schools having more than 2,800 faculty and 35,000 students. Of the total student population, 9,940 are currently enrolled in Ph.D. degree programs and 1752 are in professional schools of Medicine, Law or Dentistry. The School of Medicine was founded more than 100 years ago. There are currently 180 students pursuing Ph.D. degrees at the University of Pittsburgh School of Medicine, 113 Ph.D. graduate students in the GSPH and 17 in the Biopsychology Program in FAS.

The University of Pittsburgh Cancer Institute (UPCI) was established in 1984 to strengthen and expand cancer core and educational resources in the Western Pennsylvania region by developing new, more effective approaches to the prevention, diagnosis and treatment of cancer and by enhancing professional and lay educational programs. This is especially important because the Western PA region has the oldest population of any in the USA. Thus, the projected incidence of tumors in the female population is extremely high. In less than ten years UPCI has become the major focal point for research and education, not only in the Western PA, but also in Northern West Virginia and Eastern Ohio. It is now ranked 4th in the United States among recipients for NCI funding with more than $35 million annually. UPCI has dedicated basic and clinical research facilities totaling over 250,000 square feet for laboratory studies, 28,000 square feet for outpatient services and over 100 beds for cancer inpatients. Over 200 women are treated annually for breast cancer at UPCI. UPCI has been responsible for recruiting more than 100 cancer researchers to the Institution, including both the Training Program Director, Dr. John S. Lazo (Yale University 1989) and the Co-Director, Dr. Olivera Finn (Duke University 1991).

The University of Pittsburgh has been widely recognized since the late 1950s for its research in breast cancer largely due to the pioneering work of Dr. Bernard Fisher. His work on breast cancer metastasis defined breast cancer as an early systemic disease. This profoundly changed the treatment paradigm, which now is being translated into a choice of breast-preserving surgery over radical mastectomy. More recently, effective utilization of clinical trials, performed in multiple clinical centers, has shown effectiveness of tamoxifen in prevention of breast cancer. Magee Women's Research Institute, associated with UPCI, is one of few institutes in the world dedicated to women's care. It has achieved highly visible success in serving the needs of breast cancer patients, as well as providing samples and scientific questions for a number of researchers associated with it. UPCI also has the Vanguard Center for Women's Health Initiative, which provides screening and evaluation facilities. Women with abnormal results are channeled through UPCI or Magee Women's Breast Cancer Clinics. They account for over 500 new cases of breast cancer annually.

3.2 Graduate Training: Prior to 1997, the Ph.D. granting programs in the School of Medicine were strictly tied to Departments that had no formal interactions. We now have a new Interdisciplinary Biomedical Graduate program (INTBP). The goal of the consolidation process was to increase support from the University of Pittsburgh School of Medicine for graduate recruiting, retention and education. Our new INTBP now mandates both formal and informal interactions among the previously existing Ph.D. granting programs and allows new programs to be developed without the construction of a new department. Moreover, the INTBP has encouraged the Dean to contribute significantly more funds to

graduate education. The restructuring of the Ph.D. Programs provides greater opportunities and flexibility for most eligible incoming students. Centralized admission resources provide new opportunities to attract a broad range of potential candidates that previously were unaware of our training programs.

4. Program Faculty

The Program Director was Dr. John S. Lazo, Chair of the Department of Pharmacology and Co-Director of the UPCI Molecular Therapeutics/Drug Discovery Program. Dr. Lazo has more than 25 years of research experience in cancer biology and experimental therapeutics. Much of his early work was directed at mechanisms of drug action and drug resistance. Most of this research has been tumor type-independent in focus. He is a member of the Board of Directors of the American Association for Cancer Research and was Chair of the 1992 Gordon Research Conference on Chemotherapy of Experimental and Clinical Cancer. He has been a Ph.D. advisor for five students, has been a committee member for twenty two Ph.D. candidates and has trained twenty five postdoctoral fellows. He currently is thesis advisor for one Ph.D. candidate, who is working on issues relating to breast cancer. Two of his previous postdoctoral fellows are investigating new anticancer agents as clinical pharmacologists at a major pharmaceutical firm (Bristol Myers Squibb) and one is designing new diagnostic agents at a biotechnology company. Since 1976 Dr. Lazo has been intimately involved in both graduate and medical education. His basic medical science preparatory book for second year medical students (*Review of USMLE Step One,* Williams & Wilkins, publishers) is among the most popular books of its kind and is about to enter its fifth edition. He is also PI on an NIH Predoctoral Training Grant.

Dr. Olivera Finn served as a Co-Director of the Training Program. She is Professor of Molecular Genetics and Biochemistry and Director of the UPCI Immunology Program at the University of Pittsburgh. Dr. Finn and Dr. Lazo collaborated to couple antimucin antibodies to DNA cleaving agents and examined the role of protein phosphatase in breast cancer cells.

Faculty. The participating faculty members (Table 1) were drawn from over 175 members of the Graduate School at the University of Pittsburgh. We selected these faculty members by carefully evaluating them for excellence in the following categories: previous educational experience, suitability as a mentor, research interest in cancer (particularly in breast cancer), specific discipline to add diversity and extramural research support. We made a special effort to include a significant number of clinically trained investigators with active research programs (33% of the faculty have an M.D. degree) to ensure the appropriate exposure of students to clinically relevant issues associated with breast cancer. Participation in this Training Program was not viewed as exclusionary and new members were considered by the Training Program Executive Committee throughout the Training Program funding period.

5. Program

The study of breast cancer biology is a complex area of investigation and further understanding of this problem, as well as possible solutions, will emerge only through an influx of new investigators from many different disciplines of biology and science. The overall object of our **Training Grant in Breast Cancer Biology and Therapy** at the University of Pittsburgh was to educate students by utilizing expertise of selected faculty in Endocrinology, Pharmacology, Psychology, Behavioral Medicine, Medicine, Molecular Genetics, Immunology, Cell Biology and Epidemiology. Recruitment of these investigators from their parent departments into this Training Program was designed to support their interests in breast cancer and to provide them with an opportunity to recruit and train young investigators in the basic principles of their discipline using breast cancer as a specific model system.

Table 1. Faculty of the Training Program

Faculty	Department	Major Research Interest
John S. Lazo, Ph.D., Program Director	Pharmacology	Chemotherapy, Drug resistance, Apoptosis
Olivera J. Finn, Ph.D., Program Co-Director	Mol. Genetics & Biochemistry/Surgery	Tumor Immunology, Mucins, Immunogenetics
Edward D. Ball, M.D.	Medicine	Bone marrow transplant., Cell surface markers
Andrew Baum, Ph.D.	Psychiatry/Psychology/Beh. Neuroscience	Behavioral medicine, Stress
Robert A. Branch, M.D.	Medicine	Clinical pharmacology, Drug metabolism
Anthony R. Caggiula, Ph.D.	Psychology	Behavioral immunology, Hormones
David L. Cooper, Ph.D., M.D.	Pathology	Transcription control, Cell matrix, Gene therapy
Andrea Cortese-Hassett, Ph.D.	Pathology	Molecular genetics, Immunology
Billy W. Day, Ph.D.	Environ. & Occupat. Health/Pharm. Sci.	Molec. toxicol., Estrogen, Computational chem.
Albert D. Donnenberg, Ph.D.	Medicine	Bone marrow transplant
Maryann A. Donovan-Peluso, Ph.D.	Pathology	Molec. genetics, Transcription
Qing-Ping Dou, Ph.D.	Pharmacology	Cell cycle control, Cyclins, Transcription
Roy A. Frye, M.D., Ph.D.	Pathology	Oncogenes, Growth factors, Molec. biology
Joseph C. Glorioso, Ph.D.	Molecular Genetics and Biochemistry	Gene therapy
Ronald H. Goldfarb, Ph.D.	Pathology/Neurosurgery	Metastasis, Invasion, Proteases
Leaf Huang, Ph.D.	Pharmacology/Molec. Genetics & Biochem.	Liposomes, Gene therapy
Candace S. Johnson, Ph.D.	Otolaryngology/ Pharmacology	Exp. therapeutics, Cytokines, Vasculature
Lewis H. Kuller, M.D., Dr.P.H.	Epidemiology	Hormone metabolism, Diet, Endocrinology
Joseph Locker, M.D.	Pathology	Molecular diagnosis, Oncogenes
Michael Lotze, M.D.	Surgery/Molec. Genetics & Biochem.	Gene therapy, Immunotherapy
Susan A. McCarthy, Ph.D.	Surgery/Molec. Genetics & Biochem.	Immunology, T cell function, Apoptosis
Kenneth McCarty, M.D., Ph.D.	Pathology	Steroid receptors, Immunohistochemistry
Edward V. Prochownik, Ph.D., M.D.	Pediatrics/Molec. Genetics & Biochem.	Oncogenes, Early response genes
Paul D. Robbins, Ph.D.	Molecular Genetics and Biochemistry	Tumor suppressor genes, Gene therapy
Guillermo G. Romero	Pharmacology	Signal transduction
Herbert Rosenkranz, Ph.D.	Environmental and Occupational Health	Computational toxin analyses
Russell D. Salter, Ph.D.	Pathology	Immunology, Cell cycle proteasomes
Martin C. Schmidt, Ph.D.	Molecular Genetics and Biochemistry	Transcription factors
Said M. Sebti, Ph.D.	Pharmacology	Signal transduction, Exp. therapeutics, Drug resistance
Jill M. Siegfried, Ph.D.	Pharmacology	Growth factors, Her2/neu oncogene, Tumor vaccines
Victor Vogel, M.D.	Medicine	Biomarkers and Treatment
Theresa Whiteside, Ph.D.	Pathology/Otolaryngology	Immunology, Natural killer cells
Timothy M. Wright, M.D.	Medicine/Molec. Genetics & Biochemistry	Transcriptional regulation, Interferons
Jack C. Yalowich, Ph.D.	Pharmacology	Topoisomerases, Drug resistance, Exp. therapeutics

5.1 Predoctoral Training Pool. There are nine Ph.D. granting programs at the University of Pittsburgh School of Medicine: bioengineering (Joint Program between the School of Engineering and Medicine), Biochemistry (granted by the Department of Molecular Genetics and Biochemistry), Microbiology (granted by the Department of Molecular Genetics and Biochemistry), Pharmacology, Pathology, Cell Biology and Neurobiology Faculty of the **Training Program in Breast Cancer Biology and Therapy** are members of five of these programs. Additional faculty are from Ph.D. granting programs in the Graduate School of Public Health, Environmental and Occupational Health, FAS and Biopsychology. Admission to the Training Program requires a bachelor's degree with a major in chemistry, biology, physics, psychology, microbiology, biology or molecular biology from an accredited college or university, with a minimum GPA of 3.0.

5.2 Program Administrative Structure. The administrative structure of the Training Program used the resources of existing programs and was chaired by John S. Lazo and co-chaired by Olivera J. Finn. The routine duties such as corresponding with potential applicants, monitoring student progress, ensuring appropriate student records, distribution of information to faculty and students, seminar announcements and journal club schedules were accomplised by the Administrator of the Graduate Program, Department of Pharmacology. These individuals met on a regular basis with the Training Program Executive Committee to evaluate student progress and program needs.

5.3 Student Recruitment and Admission. The recruitment process was directed toward highly qualified students who had not yet chosen their research topic or advisor or who had identified a breast cancer related research project. Training faculty received a letter informing them of the program and announcements were posted throughout the University. Applications for admission into the **Training Program in Breast Cancer Biology and Therapy** were evaluated by the Breast Cancer Training Grant Executive Committee. This Committee included the Director and Co-Director of the Program and five other faculty members selected for their research interests and diversity. They were: Drs. Kuller, Caggiula, Siegfried, McCarty and Whiteside. Several faculty members interviewed the candidates prior to the meeting and provided information about the applicants. Applicants were judged based on their undergraduate record, results of GRE scores, performance in first and/or second year of graduate school, faculty comments and a brief written statement of their research interest as related to breast cancer. Efforts were made to ensure equitable distribution of fellowships among the represented disciplines and areas of research. The awards were granted for two years for each student, pending successful completion of the first year. This allowed the student security of funding in the final years of the project and allowed the program to bring in six new students in the third and fourth years of the grant, thereby supporting 12 students in 4 years.

5.4 Course of Study. Inasmuch as the students supported by this Training Grant belong to various programs of the INTBP, the formal course work requirements and credits of dissertation research were determined by their individual programs. The **Training Program in Breast Cancer Biology and Therapy** required that the students complete an Ethics Course offered by the University and attend the weekly conference on Breast Cancer Biology and Therapy organized every Thursday afternoon. They were also required to attend and present their work at yearly minisymposia and symposia on basic and translational research in breast cancer. These conferences were started three years ago as a collaborative effort of the Training Program and the research program in breast cancer. They were designed to highlight research of local faculty. Each student was required to present a seminar in this series at least once during the two year period of support under the training grant. These conferences also included a formal seminar series that featured outside speakers who are experts in various biological and behavioral aspects of breast cancer biology and therapy. The students were given a list of recommended courses to take as electives. The courses were chosen by the Executive Committee as most relevant to research in breast cancer. Students

wrote a progress report at the end of each year and formally presented their work in a one day program retreat on campus. Students' progress was monitored through quarterly meetings of all students with the Program Director and Co-Director and at the yearly retreats with the Executive Committee and training faculty.

5.5 Responsible Conduct of Research. Responsible conduct is an essential part of being a researcher. As competition for grants, publications and tenured positions intensifies, so does the possibility of violating the strict ethical code surrounding biomedical investigation. We believe that intensive examination of biomedical ethics is an integral part of all scientific and medical training our students receive. Upon entry into the program, all students were provided copies of *Guidelines on Academic Integrity and Research Integrity Policy*. All INTBP students must attend the Professional Skills and Ethics Class. This course is overseen by an advisory committee of students and faculty, of which Dr. Lazo is a member, as well as a team of educational evaluators. Additional, advanced training in specific areas including communication skills and teaching also is available through workshops.

5.6 Student Research. A summary of the students' progress during the entire funding of this Training Program is given on the following pages.

F. Student Research Reports

Linda Baker (Grant Recipient 1997-1998)

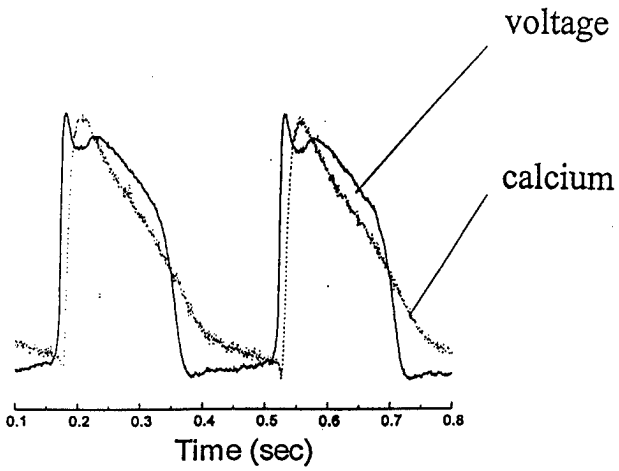
Advisor: Guy Salama, Ph.D.

Research: Role of Potassium Channels in Breast Cancer

The general goal of this research was to understand the relation between $[Ca]_i$ and membrane potential by K+ channel modification on wild type and tumor growth mice. In breast tissue and breast epithelial cells, membrane potentials were more depolarized compared with normal breast cells and were found to be particularly sensitive to the actions of K+ channel blockers. Therefore, we hypothesized that it could be possible to control the rate of cell proliferation by modification of K+ channels.

To understand the correlation between $[Ca]_i$ and membrane potential, the majority of the time was spent developing the first ever optical mapping apparatus consisting of a single excitation and dual emission. The latter is detected by two photodiode arrays to simultaneous map membrane potential and calcium transients. Cells are stained with Rhod-2 and di-4-ANEPPS, placed on the stage of an inverted microscope and excited at 520 nm. Fluorescence was collected with a 20 X objective passed through a dichroic mirror to reflect light below 640 nm and transmit light greater than 645 nm. The fluorescence from two wavelengths was focused in two 16 X 16 photodiode arrays, one measures calcium transients and the other membrane potential. Two optical probes record simultaneously, di-4-ANEPPPS to measure changes in transmembrane potential and Rhod-2 to measure cytosolic Ca2+, Cai. Both dyes can be excited at a wavelengths of 520 $\pm$ 30 nm but fluorescence at different wavelengths (di-4-ANEPPS > 665 nm, and Rhod-2 at 585 $\pm$20 nm). The photocurrent from each diode was passed through an I/V converter, a circuit to control AC-DC, a variable gain amplifier, and A/D multiplier to digitize and store the signal in the computer. Software was developed to analyze 512 channels of optical traces (256 channels per array) plus 8 channels of instrumentation. Software programs were designed to map the spread of membrane potentials and intracellular Ca2+ concentration. Simultaneous measurements of membrane potential and calcium transients can yield valuable information of understanding the importance of membrane potential in regulation of Ca2+ homeostasis.

As a rapid test of the system, voltage and Cai transients were measured from ventricular muscle where these parameters have been well characterized. As shown in the figure, cardiac action potentials and Cai can be measured with excellent signal to noise ratio from 250 X 250 micron areas. With this new optical apparatus successfully completed, we can now evaluate different potassium channel modifications on breast cells from the wild type and tumor growth. Potassium channel modification will be produced by adding $K+_{ATP}$ channel blockers, $K+_{ATP}$ channel agonist, or a Ca+-activated K+ channel blocker. In conclusion, by developing this new state-of–the-art optical mapping system we now have a very accurate and new method to determining whether cell proliferation can be controlled through the $K+_{ATP}$ channels or Ca-activated K+ channel.



An example of voltage signal and calcium transients taken simulatanously of heart tissue on the new optical mapping sytem.

Marni Brisson (Grant Recipient 1996-1997 and 1997-1998)
Advisor: Leaf Huang, Ph.D.

Research: Optimization of a novel cytoplasmic expression system

The cytoplasmic expression system was designed to address the problems of limited nuclear entry of transfected DNA. Most non-viral vectors have been optimized to increase the release of transfected plasmid DNA into the cytoplasm of cells. However, only about 1% of this DNA reaches the nucleus of the cell where the transcription machinery resides. Therefore, a higher percentage of DNA is available for cytoplasmic expression, making it more efficient in comparison to nuclear expression.

Transgenes expressed in the cytoplasm are driven by a T7 bacteriophage promoter. Transcription begins when the cell is supplied with a source of T7 RNA polymerase which recognizes the promoter. Previously, this was done by transfecting a T7 RNA polymerase autogene consisting of a T7 promoter followed by the T7 RNA polymerase gene. The autogene was co-delivered with purified T7 RNA polymerase protein which would recognize the T7 promoter on the autogene and begin transcription. The newly formed T7 RNA polymerase could then cycle back and continuously produce more enzyme. Our novel system negates the need for exogenous purified enzyme by incorporating a CMV promoter in front of the T7 promoter producing pCMV/T7-T7pol autogene. In this way, the small amount of DNA that enters the nucleus will allow transcription at the CMV promoter to produce an initial amount of T7 RNA polymerase in the cytoplasm. This will recognize the T7 promoter on pCMV/T7-T7pol and begin the autogene process. The advantages of this is avoiding the need for purified enzyme which will reduce cost and possible immunogenicity. Furthermore, our autogene is easily amplified and purified from bacteria where other autogenes require complicated and time consuming procedures.

The pCMV/T7-T7pol was compared with a previously used autogene such as pT7 AUTO 2C$^-$ in transfections with pT7-CAT. It was apparent that CAT activity was at least 4-fold higher when using the new autogene. This is due to the fact that there are overall higher levels of T7 RNA polymerase protein produced in pCMV/T7-T7pol transfected cells as determined by Western blot. CAT activity remains stable for more than 7 days after transfection, whereas CAT activity from pT7 AUTO 2C$^-$

disappears within 4 days. This is because T7 RNA polymerase protein continues to be expressed up to 5 days after transfection in pCMV/T7-T7pol transfected cells. Furthermore, transfections with pCMV/T7-T7pol and pT7-CAT demonstrate 4-fold higher CAT activity in comparison to the nuclear expression system pCMV-CAT. Therefore, the pCMV/T7-T7pol autogene represents a great improvement over the previously used cytoplasmic expression system autogenes and can even exceed expression levels from strong nuclear promoters such as CMV.

Since pCMV/T7-T7pol works as a dual autogene, it is important to determine how much of the T7 RNA polymerase protein is generated by the T7 promoter. This would prove whether or not it is working as a true autogene. For a primer extension assay, two short primers were designed to distinguish between T7 RNA polymerase message generated by the CMV promoter or the T7 promoter. The results indicate that compared to CMV-derived T7 RNA polymerase mRNA, there was greater than a 50-fold higher level of mRNA from the T7 promoter. This is extremely beneficial if this system is to work in antisense and ribozyme strategies since large amounts of mRNA can be generated in the cytoplasm. Though this system is efficient in mRNA production, when the T7 promoter is deleted in pCMV/T7-T7pol, the activity only decreases about 2 fold. This indicates that the mRNA is inefficiently translated.

We are now in the process of optimizing this system in terms of transfection formulation and improvement of the autogene itself through cloning. We are also attempting to understand the mechanism of action of this system by utilizing inhibitors at different points in the endocytotic process. It is apparent through these inhibitors that the only difference between the cytoplasmic and nuclear systems once they enter the cell is the nuclear barrier. Furthermore, when cells are arrested in G0/G1, expression levels for the nuclear system decrease about 4 fold over normal cycling cells while expression from the cytoplasmic system remains the same. When these cells are released into mitosis, expression levels increase 4 fold over normal cells while the cytoplasmic system only increases slightly. This indicates the dependence of the nuclear system on nuclear entry and the fact that the cytoplasmic expression system can avoid this problem. Therefore, the cytoplasmic system could be much more efficient in transfecting non-dividing cells such as neurons, smooth muscle, etc.

Once the cytoplasmic system with pCMV/T7-T7pol has been optimized, we can examine its effectiveness *in vivo*. Our ultimate goal is to apply this system towards cancer gene therapy. As suggested before, this system may be beneficial in antisense or ribozyme therapies. However, it could also be appropriate for other strategies such as tumor suppressor or cytokine therapies.

Publications:

Brisson M, He Y, Li S, Yang JP, Huang L. (1998) A novel T7 RNA polymerase autogene for efficient cytoplasmic expression. Submitted to *Gene Therapy*.

Li S, Brisson M, Huang L. (1996) Delivery of PCR amplified DNA fragments into cells: A model of using synthetic genes for gene therapy. *Gene Therapy* 4:449-454.

Swamy N, Roy A, Chang R, Brisson M, Ray R. (1995) Affinity purification of human plasma vitamin D-binding protein. *Prot. Express. Purif.* 6:185-188.

Swamy N, Brisson M, Ray R. (1995) Trp-145 is essential for the binding of 25-hydroxyvitamin D3 to human serum vitamin D-binding protein. *J. Biol. Chem.* 270:2636-2639.

Patent:

Huang L, Robbins P, Jaffurs D, Brisson M, Le S, Yang J-P. (Filed April 26, 1996) A cytoplasmic gene expression system which utilizes a prokaryotic RNA polymerase autogene.

Abstracts and Posters:

Brisson M, He Y, Le S, Yang JP, Huang L. (1998) A novel T7 RNA polymerase autogene for efficient cytoplasmic gene expression. Presented at the Annual Keystone Symposia on Synthetic Non-Viral Gene Delivery Systems.

deGroat WC, Brisson M, Erdman SL, Matsumoto G, Roppolo JR, Card JP, Vizzard MA (1995) Transneuronal labeling of neurons in the adult rat central nervous system (CNS) after injection of Pseudorabies virus (PRV) into the distal colon. Presented at the Society of Neuroscience Annual Meeting.

Ronna Campbell (Grant Recipient 1994-1995 and 1995-1996)
Advisor: Michael T. Lotze, M.D.

During this two-year period Ms. Campbell studied the role of IL-12 in breast cancer immunotherapy. Her work is still in progress supported through other sources.

Research: Interleukin 12 and breast cancer

Interleukin-12 is a heterodimeric cytokine which was purified from an Epstein-Barr virus transformed lymphoblastoid cell line based on it's ability to stimulate IFN-g production, activate NK cells and act as a growth factor for T and NK cells. In addition to its immunostimulatory activities IL-12 has recently been shown to possess an antiangiogenic capacity. This antiangiogenic capacity is mediated by IFN-g which is a powerful stimulator of inducible protein-10. IL-12 is produced predominantly by phagocytic cells, including macrophages and polymorphonuclear cells but has recently been shown to be produced by epidermoid carcinoma cell lines and normal keratinocytes.

We hypothesized that human breast cancer cell lines, like other epidermoid cell lines, may produce IL-12 and that this may inhibit the tumor growth by inducing both nonspecific immunostimulatory activities and angiogenic inhibition. We tested this hypothesis using various human breast cancer cell lines such as BT-20, MDA-NB-435s, ZR-75-30 under various culture conditions with and without stimulation. We measured IL-12 production by ELISA and found no IL-12 production.

Due to this absence of IL-12 production in breast cancer cell lines, we decided to further investigate IL-12 production by EBV-transformed B-cell lines and to determine if normal B-cells are capable of producing IL-12. We found that EBV-transformed B-cells express high levels of IL-12 when stimulated by PDBu (70-80 pg/ml/10^6 cells/24 hrs) and that this IL-12 production is not modulated by addition of cytokines. Furthermore, we found that human B-cells purified from peripheral blood, tonsil, or spleen did not produce IL-12 under a wide variety of stimulatory conditions and at

multiple time points. Our results are in concordance with other researchers and we plan to submit these results in collaboration with Jacques Banchereau for publication.

Albert Cunningham (Grant Recipient for 1994-1995 and 1995-1996)
Advisor: Herbert S. Rosenkranz, Ph.D.

Mr. Cunningham completed his course requirements for the Department of Environmental and Occupational Health while supported through this grant award. Also during this time he conducted research in two areas relevant to furthering the understanding of breast cancer. The first of these areas concentrated on using structure-activity relationship modeling to investigate structural features of chemicals that were associated with carcinogenesis in mice and rats. From this work, it was observed that a specific structural feature associated with carcinogenesis in mice was derived from 17-estradiol and diethylstilbestrol, two potent estrogenic chemicals. The second phase of his studies concentrated on investigating the possible role of this structural feature in the development of breast cancer from environmental estrogenic chemicals. Interestingly, at that time, chemicals with estrogenic activity were being assigned two diverse relationships with breast disease. First, man-made estrogenic chemicals with estrogenic activity (e.g., certain pesticides, plasticizers and PCBs) were implicated as causative agents for the disease. Alternately, phytoestrogens which are naturally occurring plant derived estrogenic chemicals (e.g., genistein from soybeans) were suggested as playing a preventative role in breast cancer induction. Using several independent analysis techniques, it was observed that many man-made estrogenic chemicals were characterized by a lipophilic region which is absent from non-estrogenic chemicals as well as from most phytoestrogens. It was suggested that this lipophilic region affects binding to specific receptors and may in fact serve to differentiate the so-called "harmful" from "beneficial" estrogens. Last year, Mr. Cunningham was awarded a two-year predoctoral traineeship from the Department of Defense US Army Medical Research and Material Command Breast Cancer Research Program (BCRP) to further investigate structural features of environmental estrogens. Currently, Mr. Cunningham is within a month of defending his doctoral dissertation. His future plans include staying in this Department for one year as a post-doctoral student to complete his work for the BCRP. He has tentatively accepted a three-year post-doctoral position in the Department of Cancer Cell Biology at the Harvard School of Public Health.

Publications:

Cunningham, AR, Rosenkranz, HS, Zhang, YP, Klopman, G (In press) Identification of "genotoxic" and "non-genotoxic" alerts for cancer in rats: The carcinogenic potency database, *Mut. Res.*

Cunningham, AR, Rosenkranz, HS, Zhang, YP, Klopman, G (In press) Identification of "genotoxic" and non-genotoxic" alerts for cancer in mice: The carcinogenic potency database, *Mut.Res.*

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Cheryl L. Fattman (Grant Recipient for 1996-1997 and 1997-1998)
Advisor: Q.P. Dou, Ph.D.

Introduction:

A balance between cell proliferation and cell death determines control of cell number in a tissue or organism. Apoptosis is a very specific type of cell death in which the cells essentially commit suicide. Diverse factors modulate the induction of this process. Such factors include growth factors, intracellular mediators of signal transduction, and nuclear proteins regulating gene expression, DNA replication, and the cell cycle (1). Apoptotic cells undergo a stereotypical set of changes including cell shrinkage, condensation and fragmentation of the nucleus, and cleavage of nuclear DNA that make this a unique form of cell death (2). The mechanism by which these changes occur is still largely unknown but recent investigations have indicated that a family of proteases, termed caspases, are essential components of the cell death pathway. The caspases have been shown to degrade proteins necessary to maintain the cell growth/cell death balance (3). However, disruption of this balance, through inappropriate apoptosis, can lead to such diseases as cancer or AIDS.

Relevance to breast cancer:

A variety of models have been proposed to explain the mechanism by which antiestrogens inhibit the growth of breast cancer. Many studies, focusing on the effects of antiestrogens such as tamoxifen, have indicated that these compounds act in both a cytostatic (by causing G0/G1 arrest) and cytotoxic (by inducing apoptosis) manner (4). This dual effect of tamoxifen suggests that this drug targets the checkpoint between cell cycle progression and apoptosis. One proposed mechanism for tamoxifen-triggered apoptosis involves the induction of TGF-b1, a cytokine that exhibits growth inhibitory effects in human breast cancer (4).

One critical regulator for both cell cycle and apoptotic pathways is p53, a tumor suppressor protein and a transcription factor. It has been shown that p53 regulates a DNA damage-triggered G1 checkpoint (5). In some instances, induction of p53 results in transcriptional activation of p21 (WAF1/CIP1/SDI1), leading to G1 arrest and allowing the cell to repair the damaged DNA. If the DNA damage is too severe, however, p53 induction can result in the activation of the apoptotic pathway and the demise of the cell.

The p53 gene may induce apoptosis by altering the activity of a second protein, the retinoblastoma protein (RB). Mutations of RB were first identified through examination of hereditary forms of retinoblastoma. Since then, the mutant form of RB has been associated with breast carcinomas, lung carcinomas and prostate carcinomas, among others. RB is one of the major checkpoint proteins in the cell and its activity is regulated by phosphorylation. Under normal conditions, RB is in an active, unphos-phorylated state (p110/RB), and prevents the cells from traversing the cell cycle. Conversely, inactivation of RB by phosphorylation (p120/RB), allows the cells to progress through the cell cycle (6). The p53 protein plays a role in determining the activity/inactivity of RB by regulating levels of p21, which inhibits cyclin-dependent kinase (cdk)-mediated phosphorylation of RB. However, when cells are induced to undergo apoptosis, the p120/RB is first dephosphorylated to a unique p115/RB. RB is then subsequently cleaved into three fragments, p48/RB, p68/RB, and p112/RB by a protease with properties of a caspase enzyme (7,8).

Research performed:

I have investigated the role of RB in antiestrogen-induced apoptosis of two different breast cancer cell lines: MCF-7 and MDA-MB-231. These cell lines differ in both their estrogen receptor status (MCF-7 cells express ER, while MDA-MB-231 cells do not) and their p53 levels (MCF-7 cells express wild-type p53, while MDA-MB-231 cells overexpress mutant, non-functional p53). I have shown that these two cell lines are equally sensitive to 50mM tamoxifen since both cell lines show: 1.) the characteristic morphological changes associated with apoptosis at 4h of treatment, and 2.) an increase in the pre-G1 population at 4h, indicative of DNA fragmentation. Both MCF-7 and MDA-MB-231 cells show concurrent RB dephosphorylation and caspase-mediated cleavage of RB and poly(ADP-ribose) polymerase. However, tamoxifen treatment of these cell lines did not increase the level of p53 or p21 protein, indicating that tamoxifen-induced RB dephosphorylation and apoptosis are independent of both p53 and p21 regulation.

I then investigated the role of several proteins known to be regulators of apoptosis. It has been found that the Bcl-2 oncoprotein is a ubiquitous inhibitor of cell death triggered by multiple pathways. In contrast, Bax, a Bcl-2-associated protein, promotes apoptosis by suppressing the activity of Bcl-2 (9). To examine the possibility that Bcl-2 family protein might be involved in the process of tamoxifen-induced apoptosis in human breast cancer cells, I determined the levels of Bax and Bcl-2 proteins. When MDA-MB-231 cells were treated with tamoxifen, expression of Bax protein increased over a period of 6h. In contrast, Bcl-2 expression only slightly increased over the same time period. Taken together, tamoxifen treatment increases the ratio of Bax to Bcl-2 in an early stage of apoptosis and therefore may contribute to the initiation of apoptosis in MDA-MB-231 cells. However, in MCF-7 cells treated with tamoxifen, levels of both Bax and Bcl-2 remained relatively unchanged over the 6h period. It appears, therefore, that the ratio of Bax to Bcl-2 may not play an essential role in MCF-7 cell death.

Previous studies suggest that TGF-b1 is an important negative regulator of breast cancer cell growth and that one possible mechanism of tamoxifen action is through upregulation of TGF-b1. I therefore investigated possible changes in TGF-b1 levels during tamoxifen treatment in this system. A transient induction of TGF-b1 protein was observed in MDA-MB-231 cells treated with tamoxifen for 6h, which was accompanied by increased formation of apoptotic nuclei. This data suggests that induction of TGF-b1 is associated with apoptosis in MDA-MB-231 cells. In contrast, in MCF-7 cells treated with tamoxifen, expression of TGF-b1 decreased over the same time period. It is therefore unlikely that TGF-b1 induction is responsible for apoptosis in MCF-7 cells.

Finally, increases in c-Myc levels have been associated with induction of apoptosis in some cell systems (10) including tamoxifen-treated breast cancer cells. A transient increase in levels of c-Myc protein was observed in MDA-MB-231 cells treated with tamoxifen for up to 6h. This was unexpected since a function of TGF-b1 is to downregulate levels of c-Myc protein. No such induction was found in MCF-7 cells under the same experimental conditions.

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Fattman CL, An B, Sussman L, Dou QP. (1997) Tamoxifen-induced p53-independent dephosphorylation of the retinoblastoma protein during apoptosis of human breast carcinoma cells. Abstract presented, 2nd Annual Biomedical Graduate Student Symposia.

Fattman, CL. (1997) "Dephosphorylation of RB during breast cancer apoptosis." Invited Speaker, Magee Women's Hospital.

Raymond Ganster (Grant Recipient 1994-1995 and 1995-1996)
Advisor: Martin C. Schmidt, Ph.D.

During the period that Raymond Ganster was supported by the Breast Cancer Predoctoral Training Grant he published three papers. We regret that this training grant was not acknowledged in those papers.

Research summary:

Dr. Ganster is studying the regulation of gene expression by proteins that interact directly with the TATA binding protein. The TATA-binding protein (TBP) is perhaps the single most important transcription factor in all eukaryots since it is essential for transcription initiation by all three nuclear RNA polymerase enzymes. The regulation of transcription initiation is the primary determinant of differential patterns of gene expression. Numerous proteins implicated in the control of differentiation and oncogenesis are transcriptional regulators and some have been shown to directly interact with TBP. Studies with purified TBP and p53 have shown that these proteins bind to one another and suggest that the ability of p53 to regulate gene expression may depend on its ability to bind TBP.

Ray has used yeast genetics to identify a novel protein, Std1, that binds TBP and regulates gene expression. The Std1 protein binds directly to TBP and acts as a regulator of TBP function. STD1 regulates expression of the SUC2 gene of yeast. This gene is regulated by glucose levels in the media and our data suggest that STD1 plays a key role in transducing information from the plasma membrane proteins that act as glucose sensors to the transcriptional apparatus.

Relevance to breast cancer:

All cancers are ultimately caused by cells with defects in the regulation of gene expression. Failure to express key cell cycle regulators and over expression of growth factors both contribute to oncogenic progression. Dr. Ganster's research focused on the mechanisms of signal transduction and gene regulation in yeast. Since many of proteins that regulate gene expression in yeast have mammalian homologues, studies of gene expression in yeast may identify targets for new cancer drugs.

Publications:

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Quinn J, Fyrberg AM, Ganster RW, Schmidt MC, Peterson CL. (1996) DNA-binding properties of the yeast SWI/SNF complex. *Nature* 379:844-847.

Ganster RW, McCartney RR, Schmidt MC. (1998) Identification of a calcineurin-independent pathway required for sodium ion stress response in *Saccharomyces cerevisiae*. *Genetics*, in press.

David Mark Krisky (Grant Recipient 1994-1995 and 1995-1996)
Advisor: Joseph C. Glorioso, Ph.D.

Research:

Although once thought only to be suitable for gene transfer to the nervous system, gene therapy vectors based on herpes simplex virus type 1 (HSV-1) possess unique attributes, which suggest that they hold great potential for cancer gene therapy. These attributes include the ability to infect many human cell types efficiently, the ability to be grown to high titers, and an exogenous DNA capacity far beyond other viral vectors. The work supported by this grant resulted in a number of advances in the development of replication incompetent HSV-1 gene therapy vectors. These include; one, the deletion of multiple immediate genes from the vector resulting in reduced cytotoxicity, two, the development of a rapid method for the generation of recombinant vectors, and three, the use of this system to produce vectors capable of expressing up to five separate transgenes (HSV-TK, IL-2, GM-CSF, B7.1, and IFN-g from a singe vector background. Currently these vectors are being tested for anti-tumor activity in a direct injection *in vivo* model as well as a direct injection *in vivo* vaccination model in conjunction with tumor antigens including MUC-1.

6/93-9/97. Development of replication defective herpes simplex virus gene therapy vectors. University of Pittsburgh School of Medicine. Principal investigator, Joseph C. Glorioso Ph.D.

9/97-present. Development of replication defective herpes simplex hybrid gene therapy vectors.

Publications:

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Chapters in Books:

Krisky D, Marconi P, Goins WF, Glorioso JC. (1996) Development of Replication-Defective Herpes Simplex Virus Vectors. IN: *Methods in Molecular Medicine, Gene Therapy Protocols*. P.Robbins (Ed.) Humana Press Inc. Totowa, N.J..

Goins WF, Marconi P, Krisky D, Ramakrishnan R, Fink DJ, Glorioso JC. (In press) Construction of Replication Defective Herpes Simplex Virus Vectors. IN: *Current Protocols in Human Genetics*. E. Nabel (Ed.), John Wiley & Sons, Inc.

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Abstracts:

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Moriuchi S, Oligino T, Krisky DM, Marconi P, Fink DJ, and Glorioso JC. (1998) Enhanced killing of human glioblastoma cells by TNF-a and HSV-tk expressing HSV with GCV. Abstract 342: Keystone Symposia; Molecular and Cellular Biology of Gene Therapy. Keystone, CO.

Edwina C. Lerner (Grant Recipient 1994-1995 and 1995-1996)
Advisor: Said M. Sebti, Ph.D.

Edwina Lerner graduated with a Ph.D. from Pharmacology in August of 1997. She has made major contributions to the mechanism of action of farnesyltransferase inhibitors (FTIs) as novel anticancer drugs. Her work focused on the RAS pathway in human breast cancer and the development of breast cancer specific small molecules. Dr. Lerner demonstrated that FTIs block H-Ras transformation by inducing the accumulation of cytosolic Ras/Raf complexes where Raf kinase is inactive. She then went on to show that H-Ras is much more sensitive to FTIs than K-Ras and that the relative resistance of K-Ras to FTIs is due to alternative prenylation by geranylgeranyltransferase I. Consistent with this, Dr. Lerner showed that both FTIs and GGTIs are required to inhibit K-Ras prenylation. Finally, she demonstrated that inhibition of K-Ras prenylation is not required for inhibition of human tumor growth by FTIs.

Publications:

Lerner EC, Hamilton AD, Sebti SM. (1997) Inhibition of Ras prenylation: A signaling target for novel anticancer drug design. *Anticancer Drug Design* 12:229-238.

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Amie J. McClellan (Grant Recipient 1996-1997 and 1997-1998)

Advisor: Jeffrey L. Brodsky, Ph.D.

Research: The Requirement for ATP and Specific Hsc70-DnaJ Homologue Interactions for Protein Translocation into the Yeast Endoplasmic Reticulum.

Introduction:

In order to arrive at their ultimate destinations, integral membrane proteins, secreted polypeptides, and resident proteins of most intracellular organelles must first successfully traverse the secretory pathway. Included in this range of molecules are peptide hormones, growth factors and their receptors, and enzymes necessary for homeostasis. Thus, one can see why the proper localization of the proteins that utilize the secretory pathway is necessary for normal metabolic growth. In fact, the mislocalization of certain secreted proteins has been shown to be one cause of metabolic disorders such as cancer, cystic fibrosis, heart disease, and diabetes. The first committed step in the secretory pathway is the translocation of proteins into the ER.

Relevance to breast cancer:

Proteins that traverse the secretory pathway play a necessary role in cancer growth and metastasis. For example, the invasive and metastatic abilities of tumor cells are promoted by the secretion of such molecules as collagenases, matrix-degrading proteases, and motility factors (1, 2). Also, the division of human breast cancer cells and glioblastoma cells is stimulated by the secreted growth factor IGF-1 (3, 4), and melanoma cell growth is activated by the secretion of interleukin-8 (5).

In a recent study, the non-tumorigenic EPH4 cell line, derived from spontaneously immortalized mouse mammary cells, was examined for alterations in response to thyroid hormone (T_3; 3, 5, 3 - L - triiodothyronine) (2). Investigators uncovered several actions of T_3 on mammary epithelial cell function that are likely to correspond to the development of breast cancer, including metastatic potential. The effects of T_3 included increases in the secretion of the extracellular matrix proteases stromelysin 1 and stromelysin 2 (ST-1 and ST-2), enhancement of the collagenolytic activity of the cells, and loss of cell polarity as noted by the mislocalization of several apical and basolateral proteins. All of these effects of T_3 are dependent upon the level of expression and proper secretion of the T_3 receptor, TRα-1. As protein translocation into the ER is the first committed step in protein secretion, elucidating the mechanism of protein translocation is a necessary goal preceding the design of chemotherapeutic agents to selectively inhibit the secretion of cancer promoting factors, such as TRα-1, ST-1, ST-2, and T_3. The research proposed in this application will provide a means to this end.

Another recent study examined the link between GRP78/BiP suppression and the inhibition of tumor progression (6). In B/C10ME fibrosarcoma cells, the stress response induction of GRP78/BiP correlates with resistance to cell-mediated lysis by cytotoxic T lymphocytes. Also, *in vivo* tumor progression is greatly inhibited in a B/C10ME clonal cell line stably transfected with amplified copies of a grp78/BiP antisense vector. Based upon these results, GRP78/BiP may play a role in the control of tumor cell apoptosis. GRP78/BiP was also recently noted to be highly expressed, to a statistically significant degree, in a study comparing human breast ductal carcinoma and normal breast tissue (7). If we can understand the function of BiP in the cell, we may one day be able to

alter its regulation and overcome the stress response induction that correlates with, and perhaps promotes, tumor progression.

Research performed:

In addition to the integral membrane and membrane-associated proteins that comprise the translocation machinery at the ER membrane, translocation requires the function of cytosolic and ER lumenal heat shock cognate proteins (hsc70s). Hsc70s couple ATP binding and release to repeated cycles of protein binding and release. Thus, hsc70s probably interact with and maintain translocating proteins in an unfolded and, therefore, translocation-competent state as they traverse the ER lipid bilayer. In addition, these hsc70s interact with members of the DnaJ family of molecular chaperones that are also required for translocation. Although various models have been suggested to explain how these chaperones facilitate protein translocation, their mode of action at the molecular level remains obscure. The goal of my project has been to define the role(s) of these hsc70 and DnaJ molecular chaperones during protein translocation into the ER.

To this end, I have purified the cytosolic hsc70, Ssa1p, the ER lumenal hsc70, BiP, the cytosolic DnaJ homologue, Ydj1p, and a GST-fusion protein containing the ER-lumenal DnaJ-like domain of the ER integral membrane protein Sec63p (GST-63Jp). I have found that while Ssa1p and BiP exhibit identical rates of ATP hydrolysis in a steady-state kinetic analysis, the ATPase activity of Ssa1p is stimulated by Ydj1p up to 10-fold, but Ydj1p stimulated the ATPase activity of BiP less than 2-fold. Conversely, GST-63Jp specifically stimulated the ATPase activity of BiP, but not Ssa1p. Additionally, in a native gradient PAGE assay, Ydj1p and ATP elicited the release of a radiolabeled unfolded protein substrate, ^{125}I-CMLA, from Ssa1p, but not from BiP. The specificity of DnaK homologue DnaJ homologue interactions was further established by showing that BiP, but not Ssa1p, stably binds to GST-63Jp in an ATP-dependent manner. Therefore, the specificity of these hsc70s for their compartment-specific DnaJ homologues is the basis of their topologically-restricted functions in protein translocation.

To identify essential functions of BiP, four dominant lethal mutants of BiP have also been purified. All four mutant proteins are the result of single amino acid substitutions in the ATPase domain. Because it is known that BiP forms an ATP-dependent complex with the translocation machinery via the DnaJ-domain of Sec63p, BiP and ATP hydrolysis may regulate protein translocation. I have discovered that the dominant lethal mutants of BiP are essentially devoid of ATPase activity in comparison to wild type BiP, although they are able to bind ATP as determined by Lineweaver-Burk analysis of steady-state ATPase assays. The mutant BiP proteins are also unable to associate with GST-63Jp in an ATP-dependent manner. The ability of the BiP mutants to bind to an unfolded protein substrate, ^{125}I-CMLA , does not appear to be compromised. However, the mutant proteins may be defective for release of protein substrate, but an assay demonstrating the release of unfolded protein substrates from wild type BiP has not yet been established. Most interestingly, all four dominant lethal BiP mutants inhibit the translocation of the yeast mating pheromone precursor, ppαf, in *in vitro* translocation assays. This inhibition of translocation is observed using both equimolar amounts of wild type and mutant protein, and when wild type protein is present in great excess. As wild type and all four mutant proteins exist as monomers and dimers when assessed by native PAGE, perhaps nonfunctional wild type mutant dimers are formed *in vivo* and this results in the lethal phenotype.

In addition, I have collaborated with the laboratory of Professor James Pipas in this department and demonstrated that the J-domain of SV40 T antigen is able to functionally interact with Ssa1p. That

is, the T antigen J-domain stimulates the ATPase activity of Ssa1p, and, in combination with ATP and Mg^{2+}, T antigen effected the release of Ssa1p from ^{125}I-CMLA. SV40 T antigen has been shown to transform mammalian cell lines, and is known to interfere with the normal cellular function of members of the retinoblastoma family of proteins and the tumor suppressor p53. The biochemical assays I have established can differentiate between functional and non-functional T antigen J domains, may lead to new discoveries regarding the mode of action of SV40 T antigen in tumorigenesis, and provide a new means to test the efficacy of drugs designed to inhibit the activity of T antigen.

In summary, these and future experiments will serve to elucidate the role(s) of hsc70s in the translocation of proteins across the ER membrane and in cellular physiology. This information will further clarify the mechanism of protein secretion and may aid in the development of new therapies and diagnoses for diseases that result from the mislocalization of proteins that utilize the secretory pathway.

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Vartikar A, McClellan J, Srinivasan A, Cantalupo A, Kelley P, Marks W, Li I, Whyte Y, P, Rundell K, Georgopolous C, Brodsky J, Pipas JM. (1998) Role of the T Antigen Chaperone Activity in SV40-Induced Tumorigenicity. Cold Spring Harbor Pathways to Cancer Meeting.

Brodsky J, Vartikar J, McClellan A, Srinivasan A, Cantalupo P, Kelley W, Georgopolous C, Tremblay J, Lewis J, Pipas JM. (1998) Large T Antigen Chaperones SV40 Growth and Tumorigenesis. Cold Spring Harbor Molecular Chaperones and the Heat Shock Response Meeting.

McClellan AJ, Pipas JM, Brodsky JL. (1998) The Amino-Terminus of SV40 T Antigen is a Functional DnaJ Chaperone. University of Pittsburgh Cancer Institute Annual Scientific Retreat.

McClellan AJ, Endres JB, Brodsky JL. (!997) The requirement for ATP and specific hsc70-DnaJ homologue interactions for protein translocation into the yeast endoplasmic reticulum. University of Pittsburgh Cancer Institute Annual Scientific Retreat.

McClellan AJ, Endres JB, Brodsky JL. (1997) The requirement for ATP and Specific hsc70-DnaJ Homologue Interactions for Protein Translocation into the Yeast Endoplasmic Reticulum. Mid-Atlantic Yeast Meeting.

Vartikar V, McClellan A, Srinivasan A, Cantalupo P, Kelley W, Marks I, Brodsky J, Pipas JM. (1997) The Amino-Terminal Region of SV40 Large and Small T Antigens Functions as a J-Domain. DNA Tumor Viruses Meeting.

McClellan AJ, Endres JB, Brodsky JL. (1997) The Requirement for ATP and Specific hsc70-DnaJ Homologue Interactions for Protein Translocation into the Yeast Endoplasmic Reticulum. American Society for Cell Biology Annual Meeting.

Meetings Attended:

Mid-Atlantic Yeast Meeting, June 18-20, 1997, Pittsburgh, PA.

University of Pittsburgh Cancer Institute Annual Scientific Retreat, July 21-22, 1997, Johnstown, PA.

American Society for Cell Biology Annual Meeting, December 13-17, 1997, Washington DC.

University of Pittsburgh Cancer Institute Annual Scientific Retreat, July 16-17, 1998, Johnstown, PA.

Jennifer L. Siegert (Grant Recipient 1996-1997 and 1997-1998)
Advisor: Paul D. Robbins, Ph.D.

Research: Characterization of the Functional Interaction between the Retinoblastoma Tumor Suppressor and the TAF$_{II}$250 Transcription Coactivator.

The retinoblastoma protein, Rb, is a tumor suppressor whose mutational inactivation has been implicated to play a role in a variety of sporadic and familial human cancers such as bladder, cervical, and breast carcinomas. Rb is a key regulator of cell growth and differentiation, and although the exact mechanism by which it acts as a tumor suppressor is unclear, the ability of Rb to regulate transcription in a cell-cycle dependent manner is likely to be central to its function. Dr. Robbins' laboratory has recently demonstrated that Rb is able to bind directly to TAF$_{II}$250, the largest subunit of the TFIID basal transcription factor. The "pocket" domain of Rb, which is a hot-spot for mutations associated with breast carcinomas and other tumors, binds within a recently-characterized kinase domain in TAF$_{II}$250. In addition, both the pocket and amino terminus of Rb bind within or near to another recently characterized domain for histone acetyltransferase activity in TAF$_{II}$250.

Ms. Siegert has performed a series of kinase assays with purified recombinant proteins and has demonstrated that Rb, specifically the pocket, is able to inhibit the kinase activity of TAF$_{II}$250. Furthermore, two different tumor-associated mutations within the Rb pocket rendered Rb functionally dead for TAF$_{II}$250 kinase inhibition. The inhibition of TAF$_{II}$250 kinase activity could represent a novel mechanism of transcriptional regulation by Rb, in that it has been recently demonstrated that TAF$_{II}$250 kinase activity is important for transcription of at least some genes *in vivo*, particularly those involved in regulation of cell cycle progression and growth control. Currently, Ms. Siegert is continuing to characterize this functional interaction between Rb and TAF$_{II}$250, specifically the biological role, mechanism, and regulation of TAF$_{II}$250 kinase inhibition by Rb. She has preliminary data suggesting that cyclin D1, which binds to both TAF$_{II}$250 and Rb and is known to modulate Rb activity during the cell cycle, is able to completely block TAF$_{II}$250 kinase inhibition by Rb. Interestingly, cyclin D1 is frequently overexpressed in human breast cancers and may be contributing to the development and progression of some breast carcinomas, perhaps due in part to its inactivation of at least one of Rb's transcription-regulatory pathways. In addition to the kinase activity of TAF$_{II}$250, Ms. Siegert would also like to determine whether Rb is able to affect TAF$_{II}$250 acetyltransferase activity, as this activity is also thought to be involved in transcription regulation. It is possible that such knowledge will provide a specific functional link between the inactivation of Rb or overexpression of cyclin D1 and the onset of tumor formation.

Publications:

Siegert, JL, Rushton, JJ, Robbins PD. (In preparation) Regulation of transcription by the Rb and p53

Siegert JL, Robbins PD. (Submitted) Rb inhibits the intrinsic kinase activity of the TBP-associated factor, TAFII250.

Shao Z, Siegert JL, Ruppert S, Robbins PD. (1997) Rb interacts with TAFII250/TFIID through multiple domains. *Oncogene* 15:385-392.

Robbins PD, Shao Z, Adnane J, Siegert JL. (1995) Transcriptional regulation by the retinoblastoma tumor suppressor protein. *Molec. Cells* 5:529-538.

Presentation:

Siegert JL, Robbins PD. (1997) The retinoblastoma protein inhibits the intrinsic kinase activity of the TBP-associated factor, TAFII250. *Cancer Genetics & Tumor Suppressor Genes*, FACSI Conference, Frederick, MD.

Posters:

Siegert JL, Rushton JJ, Robbins PD. (1998) Cyclin D1modulates the functional interaction between the retinoblastoma protein and the TBP-associated factor, TAFII250. *Cancer Genetics & Tumor Suppressor Genes*, Cold Spring Harbor Laboratory, NY.

Siegert JL, Robbins PD (1997) The retinoblastoma protein inhibits the intrinsic kinase activity of the TBP-associated factor, TAFII250. *Mechanisms of Eukaryotic Transcription*, Cold Spring Harbor Laboratory, NY.

Siegert JL, Shao Z, Ruppert S, Robbins PD. (1996) Targets for the retinoblastoma tumor suppressor within the basal transcription initiation complex. *The Cell Cycle*, Keystone Symposia on Molecular & Cellular Biology, Taos, NM.

James T. Snyder (Grant Recipient 1996-1997 and 1997-1998)
Advisor: Olivera Finn, Ph.D.

Research: Deregulated expression of protein kinase C and its effects on T-lymphocyte development, responsiveness, and immune surveillance of tumors

Malignant transformation of tissues leads to reexpression of proteins that are normally expressed only transiently during development and are not found in fully differentiated tissues. These reexpressed proteins may potentially then be recognized by the immune system and serve as tumor specific antigens. In this concept of immune surveillance, tumors may arise spontaneously at various times, but they are recognized and eliminated by the immune system before they progress to a malignant state. In my project, we are testing a general hypothesis that this immune surveillance occurs, and could be viewed as a specialized form of autoimmunity.

In order for an immune response to occur, a T cell must receive a signal through its antigen specific T cell receptor. This T cell receptor signal is mediated, at least in part, by protein kinase C. We thus reasoned that disruption of this signaling process, by disregulating expression of protein kinase C at different stages of T cell development, may result in either enhanced or diminished immune surveillance of tumors. We therefore developed a test system for the following specific hypothesis:

Hypothesis: Disruption, via disregulated expression of protein kinase C (PKC), of the normal signaling processes involved in development or activation of T cells, may result in changes in the immune surveillance of tumors.

We have developed lines of transgenic mice in which expression of PKC-betaII, one of the predominant PKC isoforms found in T cells, is deregulated at different stages of T cell development. In one of these transgenic mouse lines, PKC-betaII is expressed from the *lck* proximal promoter, which will result in deregulated expression of PKC in immature T cells developing in the thymus. In another transgenic mouse line, PKC-betaII is expressed from the *lck* distal promoter, which will result in deregulated expression of PKC in mature, peripheral T cells. It was our expectation that disregulating PKC expression in this manner would result in disruption of the normal signaling processes in the T cells, which would in turn result in changes in immune competence, lack of immune surveillance, and failure to eliminate tumors. Alternatively, if these changes result in enhanced T cell function, we may have mice which are hyperimmune or autoimmune. In this case, the mice would serve as a model of tumor immunity as it relates to autoimmunity.

Progress to date: Observations on PKC transgenic mice.

In the line of mice in which PKC betaII is expressed from the *lck* proximal promoter, which should result in disregulated expression of PKC in the earlier stages of T cell development in the thymus, a number of phenotypic changes have been observed. As expected, at least in some of these mice, there are changes in thymocyte populations. We have observed skewing away from the CD4 CD8 double positive population, which is normally the predominant population in thymus, towards the double negative or single positive populations. The most frequently observed phenotype in all mice from this line is abnormal trafficking of lymphocytes into various tissues, such as liver and kidney. This may reflect changes that occurred during the development of these cells that resulted in alterations in their homing pattern. There is no evidence of any autoimmune responses in these mice, i.e.; the tissues do not show evidence of autoimmune destruction on histology. Along with the observation of lymphocyte trafficking into peripheral tissues, we see diminished numbers and percentages of lymphocytes (both T cells and B cells) in the peripheral lymphoid organs, notably the spleen. This may reflect changes in the homing pattern of these cells, or it may reflect a generalized immune deficiency. Interestingly, in this regard, these mice develop tumors of different types, with a much higher frequency than normal, age-matched controls. The tumors occur more frequently in the younger transgenic mice of this line than in normal mice, and the tumors are far more aggressive than in normal mice. We believe these changes occur due to an immune deficiency that results in reduced immune surveillance of tumors. Thus, as we expected, disruption of the normal signaling processes during T cell development, by disregulating expression of PKC, results in a diminished ability of these mice to eliminate tumors. These mice may thus serve as a good model of diminished immune surveillance of tumors.

The other line of transgenic mice, in which PKC-betaII is expressed from the *lck* distal promoter, which results in deregulated expression of PKC in mature, peripheral T cells (as well as B cells), has

a somewhat different phenotype from the line discussed above. A general finding in these mice is enhanced responsiveness of T cells to mitogenic stimuli (such as Con A) compared with normal, age-matched controls. Some of the older founder mice, as well as homozygous mice from this line, have abnormally enlarged lymph nodes. We have observed increased numbers and percentages of T cells and B cells in these mice. On histology, we observe abnormal lymphocyte trafficking into peripheral tissues, such as liver, kidney, and pancreas. This trafficking is often accompanied by evidence of autoimmune destruction of the tissues. Thus, this may be a model of a "hyperimmune" mouse, which has difficulty shutting off an immune response once it has started. As related to tumors and tumor surveillance, tumors are not generally seen as frequently in these mice as in the other transgenic line or in normal older mice.

Conclusion: We have developed two lines of transgenic mice which have disrupted T cell receptor signaling due to deregulated expression of PKC-betaII, a critical intermediate in the T cell receptor signaling pathway. One of these lines, in which expression is deregulated during T cell development, shows evidence of immune deficiency and decreased immune surveillance of tumors. The other line, in which PKC expression is deregulated in mature lymphocytes, shows evidence of immune hyperresponsiveness. These lines may prove to be good models to study the immune response to tumors and tumor specific antigens.

Manuscripts in preparation:

Snyder JT, Subbotin VM, Finn, OJ. Abnormal lymphocyte trafficking and diminished immune surveillance of tumors in transgenic mice with deregulated expression of protein kinase C betaII in immature thymocytes.

Snyder JT, Subbotin VM, Finn OJ. Hyperimmune responsiveness in transgenic mice with deregulated expression of protein kinase C betaII in mature B and T lymphocytes.

Diane M. Zeleski (Grant Recipient 1994-1995 and 1995-1996)
Advisor: Guillermo Romero, Ph.D.

Research: Insulin receptor substrate 1 (IRS-1) expression and function in human breast cancer cell lines.

Ms. Zeleski was awarded the US Army Department of Defense Predoctoral Training Grant for a two-year period from September 1994 through August 1996. Her primary research hypothesis was that a calculated reduction in IRS-1 protein levels could reverse the tumorigenicity of breast cancer. She defended her Masters dissertation in August 1998 and is employed as a Senior Clinical Research Assistant with Pharmaceutical Resource Corporation, Philadelphia, PA.

The insulin receptor (IR) is a member of the tyrosine kinase growth factor receptor family. Receptor tyrosine kinases play a key role in both normal and neoplastic cell growth. IR over-expression induces a ligand-dependent transformed phenotype. A six-fold overexpression of structurally and functionally normal IR content was reported in human breast cancer specimens as well as a number of cultured breast cancer cell lines. Additionally, IR content has been found to correlate with other clinical parameters, including tumor size and grade, that reflect increased tumor aggressiveness. Since the receptor number has been established to be aberrant, the possibility exists that by

short-circuiting the signal transduction pathway just proximal to the receptor, a reversal of the tumorigenicity of these breast cancer cell lines may be realized.

A potential candidate that fulfills the aforementioned criteria is IRS-1. Numerous studies have reported that IRS-1 is an important phosphoprotein central to many of insulin's and IGF-1's signaling pathways, and it functions just proximal to the receptor. Fortunately, the rat, mouse, and human IRS-1 sequences have recently been cloned. The three protein sequences are highly conserved (>90%), particularly in their potential phosphorylation sites. This high degree of conservation across species supports the notion of the central importance of IRS-1 in both insulin and IGF-1 signaling. IRS-1 contains at least a dozen tyrosine residues and nearly 50 serine/threonine residues as potential phosphorylation sites. Although the *in vivo* phosphorylation status of IRS-1 has not been thoroughly characterized, the enormous number of such sites implies that it is a highly regulated phosphoprotein in downstream modulation of growth factor activation. Immediately following insulin/IGF-1 stimulation, IRS-1 is rapidly phosphorylated on tyrosine residues. Tyrosyl phosphorylated IRS-1 is known to interact with the SH2 domains of several critical signaling proteins. These include: the regulatory 85 kD α subunit of PI3K; the tyrosine phosphatase, SHPTP2; the adaptor protein, Grb-2, essential for p21ras activation; and oncogenic adaptor proteins, such as Nck. An observation of considerable importance is the fact that the functional expression of IRS-1 protein has recently been established as an absolutely essential component for the mitogenic response in a CHO cell line. Given the fact that both insulin and IGF-1 stimulate proliferation of breast cancer cells and IRS-1 is a central component of this signaling, the study of the role of IRS-1 in the pathobiology of breast cancer is of considerable interest.

Preliminary studies focused on IRS-1 knockout experiments utilizing antisense strategies in a murine NIH3T3-derived fibroblast cell line, F442A. Antisense constructs were generated in the established eukaryotic expression vector, pcDNA3 (In Vitrogen), carrying antisense (or sense) versions of the rat IRS-1 cDNA (78%) or a synthetic 57-mer targeted at the initiation site. This vector is equipped to produce high-level constitutive transcription from mammalian enhancer/promoter sequences (CMV). In addition to other features not mentioned, it contains the neomycin resistance gene for selection of G418-resistant stable eukaryotic clones. The orientation of the insert in these constructs has been verified by restriction enzyme digestion and automated sequencing of the constructs. Sense orientation, as well as transfection performed with the vector alone, served as negative controls. pcDNA3 and its precursor vector, pcDNA1/neo, were successfully employed for the selection of stable transfectants by several laboratories. In addition, successful episome-based antisense strategies were reported against IRS-1 to ablate the insulin-induced mitogenic response in a stable CHO cell line, as well as against IGF-1, which resulted in a loss of tumorigenicity of rat glioblastoma. Cationic liposome-mediated transfection (Lipofectamine, Gibco, BRL) were completed in the F442A cell line, and selection and propagation of single clones stably expressing the constructs was achieved. Analysis by SDS-PAGE, Western blot, and densitometry revealed that the antisense constructs exhibit a 52% (cDNA) and 27% (57-mer) reduction in IRS-1 protein content relative to the vector control.

These preliminary studies were extended to several breast cancer cell lines. Four of the initial eight breast cancer cell lines screened were retained for further studies based upon basal IRS-1 content (W. blot) and estrogen receptor (ER) status, an important indicator of the success of breast cancer therapy. The cell lines examined were: (1) BT20 (low IRS-1, ER+ but non-functional); (2) SKBR3 (moderate IRS-1, ER to be determined); (3) T47D (moderate IRS-1, ER+); and (4) MDA-MD-231 (high IRS-1, ER-). Since Ms. Zeleski focused on mitogenic assays, these cell lines were grown in

RPMI media (+10% FBS, +1% penicillin/streptomycin) minus phenol red, which has been shown to exhibit estrogen-like effects on estrogen-sensitive cell lines. Based on the preliminary Western blots performed to determine IRS-1 content, an apparent shift in mobility of IRS-1 was noticed in these breast cancer cell lines compared to the typical mobility of the basal state. Similar mobility shifts have been observed with insulin treatment in other systems and are consistent with tyrosyl-phosphorylated activated IRS-1. The mitogenic effect of activating the insulin signal transduction pathway plays a pivotal role in the pathobiology of certain breast cancers, and ultimately, IRS-1 could be targeted effectively for gene therapy of those breast cancers. Experiments performed in the second year of funding included transfection of the breast cancer cells with the aforementioned antisense and sense constructs.

Abstract:

Yalowich JC, Allan WP, Shifko R, Zeleski D, Claycamp HG, Quinn P, Stoyanozsky D, Kagan VE. (1994) Two conflicting effects of etoposide on DNA: damage and protection against oxidative damage. Proc. Amer. Assoc. Can. Res. 35:2291.

Posters:

Zeleski, DM, Romero GG. (1996) Insulin receptor substrate 1 function and expression in human breast cancer cell lines. Presented at the First Annual Biomedical Student Research Symposium, University of Pittsburgh, Pittsburgh, PA.

Zeleski, DM, Romero GG. (1996) Insulin receptor substrate 1 function and expression in human breast cancer cell lines. Presented at Student Scientific Day, University of Pittsburgh, Pittsburgh, PA 1996.

Zeleski DM, Romero GG. (1997) Disruption of insulin responsiveness in IRS-1 antisense-expressing clones of the preadipocyte cell line, 3T3-F442A. Presented at the Second Annual Biomedical Student Research Symposium.